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DNA barcoding of Naididae (Annelida, Oligochaeta) based on COI and ITS2 genes in China

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Abstract

Exploring the effectiveness of DNA barcoding in species identification is prerequisite for biodiversity conservation and environmental monitoring. Aquatic oligochaete could serve as an excellent indicator in aquatic monitoring programs. However, few studies have examined the effectiveness of DNA barcoding in these specific organisms. The mitochondrial COI gene and nuclear ITS2 gene of 83 specimens belonging to 36 species of 18 genera were sequenced in this study. The results showed that there was a barcode gap between species of Naididae, and the intraspecific genetic distances of each species were smaller than interspecific genetic distances. The classification results of ABGD (Automatic Barcode Gap Discovery) were consistent with those of morphological identification except for *Tubifex tubifex* and *Lumbriculus variegatus*. All species were successfully distinguished in the phylogenetic tree based on ITS2 gene, which was coincident with morphological result. Our results provided evidence that DNA barcoding can be used as an effective and convenient tool for species identification of the family Naididae and even aquatic oligochaete.

Keywords

aquatic oligochaete, Naididae, DNA barcoding, COI, ITS2

Introduction

The family Naididae, as the most diverse family within the class Oligochaeta, includes more than 1100 valid species (Timm 2017). The Naididae is widely distributed in surface freshwater, groundwater and oceans around the world (Martin et al. 2007). There are nine known subfamilies of Naididae worldwide, among which four subfamilies are common in China, including Naidinae, Tubificinae, Rhyacodrilinae, and Pristininae (Wang and Cui 2008). As an excellent bioindicator, the Naididae possess desirable characteristics:

tolerance to pollution (Kračun-Kolarević et al. 2015, Xu et al. 2013), diverse reproductive strategies (Christensen 1984), limited mobility, and their ease of collection.

Rapid and unambiguous identification of species is an essential prerequisite for environmental monitoring and biodiversity (Cheng et al. 2011, Xiao et al. 2004). Both experienced taxonomists with solid professional knowledge and a relatively intact biological specimen are necessary for traditional morphological identification. However, experts that engaged in taxonomic research continue to decline. The main identification characteristics based on genitalia constitute a major obstacle to the identification of immature species. For example, two species, *Limnodrilus hoffmeisteri* Claparède, 1862 and *L. claparedianus* Ratzel, 1868 can be identified only when the specimens are in a mature state (Yasuda and Okino 1988). In addition, traditional methods are still limited to high cost and an immense amount of time. With the rapid development of molecular biology, the molecular research of species identification comes into being.

DNA-based identification methods can not only quickly and accurately identify morphologically damaged specimens (Carvalho et al. 2015), but also effectively identify species at various developmental stages (Casiraghi et al. 2010) without relying on professional morphological taxonomists. Thereinto, the cytochrome C oxidase subunit I (COI) of mitochondrial gene is ideal gene target to be used as DNA barcode for species identification due to maternal inheritance with moderate sequence conservatism and variability (Hebert et al. 2003a, Hebert et al. 2003b, Remigio 2003). DNA barcoding through a short and standardized fragment of COI gene to identify and classify species has been demonstrated in multiple taxa (Amer 2021, Kheirallah 2021, Rewicz et al. 2021, Foottit et al. 2008, Costa et al. 2007, Ball et al. 2005, Zhou et al. 2007). The technique allows identification of animal groups at the species level, as well as helps in the discovery of cryptic species (Lin et al. 2020, Ardura et al. 2010). James et al. (2010) revealed that cryptic diversity of *Lumbricus terrestris* on the basis of DNA barcoding. However, DNA barcoding based on COI gene alone could be overestimate the number of species in the species delimitation of some groups of aquatic oligochaetes (Achurra and Erséus 2013, Martinsson et al. 2013, Dasmahapatra et al. 2010). Hence, the COI gene may be not suitable for phylogenetic analysis as a single molecular marker (Zhou et al. 2010). Besides, internal transcribed spacer 2 (ITS2) of nuclear gene was often used to serve as a complementary strategy to COI in phylogenetic analysis (Envall et al. 2012). The effectiveness of this integrative method (COI and ITS2) has been demonstrated in the identification of various taxa of oligochaete, such as *Rhynchelmis* (Zhou et al. 2010), *Stylodrilus heringianus* (Achurra and Erséus 2013), *Rhyacodrilus falciformis* (Martinsson et al. 2013), *Grania* (De Wit and Erséus 2010), *Enchytraeus albidus* (Erséus et al. 2019), *Aporrectodea longa* (Martinsson et al. 2015) and so on.

Recent advance in developed countries have been established a national barcode database of aquatic oligochaete (Vivien et al. 2017), which was evidenced as useful tool in conserve biodiversity and environmental monitoring (Vivien et al. 2015). However, this knowledge is still lacking in China, despite embodied with many endemic oligochaetes (Peng et al. 2017, Peng et al. 2014, Cui et al. 2015, Cui and Wang 2012a, Cui and Wang 2012b). Considering that differences in regional species pool between different climate

zone, we should not directly extrapolate the genetic information. In this case, establishing a regional database of aquatic oligochaete is necessary to future monitoring.

In this study, we sequenced the COI and ITS2 sequences of 83 specimens under 36 species belonging to 18 genera to study the barcoding of Naididae, aiming to explore the accuracy of DNA barcoding technology for the species identification, and to construct a bio-identification system for Naididae.

Material and Methods

Specimen collection

The specimens were collected between 2017 and 2020 in China (Suppl. material 1). The anterior of material was fixed with 10% formalin as morphological evidence, and the rest of the same worm was preserved in absolute ethyl alcohol for future molecular studies.

DNA extraction and PCR amplification

Total genomic DNA was extracted using the TIANGEN blood tissue kit, following the manufacturer's instructions strictly (TIANGEN Blood and Tissue Handbook). Approximately 658 bp were amplified of the COI gene using universal primers, LCO1490-GGTCAACAAATCATAAAGATATTGG and HCO2198-TAAACTTCAGGGTGACCAAAAAATCA (Folmer et al. 1994, Bely and Wray 2004). The 25 µl PCR reaction mixes included 12.5 µL of Q5 Polymerase, 2.5 µL of 10 µmol/L of primer pair mix, 2 µL of DNA template and 8 µL ddH2O. The PCR procedures comprised an initial pre-denaturation step at 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 45°C for 45 sec and elongation at 72°C for 45 sec, with an ultimate elongation at 72°C for 3 min. The recently designed specific primers of ITS2 (606F-GTCGATGAAGAGCGCAGCCA and 1082R-TTAGTTTCTTTTCCTCCGCTT) for aquatic oligochaete were chosen (Liu and Erséus 2017). The annealing temperature of ITS2 gene was 4°C, and the other conditions were the same as that of COI gene. 5 µL PCR products were detected by 1% agarose gel electrophoresis, and the remaining products were sent to I-congene Ltd. (Wuhan, China) for sequencing.

Genetic analyses

Raw sequences were calibrated in BioEdit and assembled in SeqMan (DNASTAR). All sequences were aligned by ClustalW using MEGA. The new acquired COI sequences in our study were compared to Genbank (NCBI) sequences using BLAST. If the similarity (Per. Ident) with the target species is more than 98%, it can be used for subsequent analysis.

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Sequence composition analyses

All of COI sequences were imported into MEGA for multi-sequence alignment, and base composition, conserved sites, variable sites, parsimony informative sites, and transitions/ transversions were calculated, respectively.

Genetic distance analyses

The uncorrected pairwise genetic distances between sequences were obtained with MEGA, including various taxonomic levels, species, genus and subfamily.

Automatic barcode gap discovery (ABGD) analyses

The genetic distance matrix of all Naididae specimens was calculated using the Kimura two parameter (K2P) model (Kimura 1980). The analysis of the division of taxa was carried out on the ABGD website (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html). The prior intra-specific divergence (P) was between 0.001 and 0.1, and the minimum relative gap width (X) was 1.0, with the remaining parameters leaving default.

Phylogenetic analyses

The Bayesian trees were generated using the software Phylosuite1.2.1 (Zhang et al. 2019) to provide a graphic showing the genetic divergence between species. The optimal substitution model was selected based on BIC standard (Kalyaanamoorthy et al. 2017), with GTR+F+I+G4 for both COI and ITS2 genes. Bayesian analysis included 2 million generations, and the posterior probability indicated the confidence of each clade of the phylogenetic tree. Phylogenetic tree was submitted to iTOL website (https://itol.embl.de/itol.cgi) for online editing, then the tree graph would be exported to modify in Adobe Illustrator.

Results

Barcode sequence composition analyses

A total of 36 species belonging to 18 genera were analyzed, giving altogether 83 COI sequences. Ten of them were acquired for the first time, including *Tubifex laxus* Peng, Wang & Cui, 2017; *T. conicus* He, Wang & Cui, 2012; *Isochaetides palmatus* He, Cui & Wang, 2012; *Dero dorsalis* Ferronière, 1899; *Haemonais waldvogeli* Bretscher, 1900; *Nais simplex* Piguet, 1906; *N. inflata* Liang, 1963; *N. badia* Peng, Wang & Cui, 2014; *N. longidentata* Cui, He, Peng & Wang, 2015 and *Rhyacodrilus sinicus* (Chen 1940).

The aligned length for COI gene used in the study was 658bp, and the sequences were AT-biased (60.8%). There were 337 conserved sites and 321 variable sites, of which 319 were parsimony informative. The value of transitions/ transversions was 1.35.

Genetic distance analyses

The average genetic distances between species range from 7.6% to 28.0%. Among them, *N. longidentata* and *N. communis* having the smallest inter-specific distances, while *Limnodrilus grandisetosus* and *Lumbriculus variegatus* having the largest.

The mean intra-specific genetic distance was 0.0-20.7%, and *T. tubifex* was maximum. The maximum inter-specific genetic distance within genera were *Tubifex tubifex* and *T. conicus* (24.6%), and the minimum was between *N. longidentata* and *N. communis* (7.6%). Mean divergence among species within genera increases to 17.9%, and within subfamilies it increases to 18.9% (Table 1). Within the same subfamily, the genetic distance between *N. communis* and *U. uncinata* of Naidinae was the minimum (8.7% only), and the genetic distance between *L. grandisetosus* and *T. conicus* of Tubificinae was the maximum (25.6%).

Overall, our results showed that more variation among species than intraspecific differences (Fig. 1).

ABGD analyses

The partition results based on ABGD method included recursive partition and initial partition, and the latter was relatively stable. The COI sequences were grouped into 40 taxa (Fig. 2). Compared with the morphological results, we found that *T. tubifex* were sorted into 3 groups, and *Lumbriculus variegatus* were also divided into 3 groups, as well as the remaining 77 specimens were separated into 34 groups, which were consistent with the morphological results.

Phylogenetic analyses

The phylogenetic analysis of family Naididae based on ITS2 gene was constructed by Bayesian inference (Fig. 3). Each species was well separated from each other. All Naididae sequences clustered together in the tree based on ITS2 gene. The Naididae was monophyletic, which mainly consisted of three parts: Tubificinae, Rhyacodrilinae and Naidinae. Within the subfamily Tubificinae, the genera *Aulodrilus*, *Limnodrilus*, *Ilyodrilus* and *Potamothrix* were monophyletic and well supported (BI, 1.00). There were two genera in the subfamily Rhyacodrilinae, namely, *Bothrioneurum* and *Rhyacodrilus*. However, these two taxa did not branch together, and the genus *Rhyacodrilus* branched with the other Naidinae species. Hence, the subfamilies Rhyacodrilinae and Naidinae appeared as polyphyletic. The genus *Nais* was non-monophyletic, founded in two clades (BI, 0.97). One clade comprised *N. badia*, *N. elinguis*, *Stylaria fossularis*, and *Slavina appendiculata* and the other consisting of *N. simplex*, *N. longidentata*, *N. communis*, *N. inflata*, *N. pardalis*, *N. bretcheri* and *Uncinais uncinata*. The genus *Tubifex* was not monophyletic.

Discussion

The core principle of DNA barcoding is that inter-specific genetic distance is greater than intra-species difference (Hebert et al. 2003a). The genetic distance increases with the distance of the relationship. In this study, there was more variation among species of the same subfamily than among congeneric species. The genetic divergence among species of the same genus was greater than that conspecific individuals. Overall, the distance between species was greater than the intra-species distance, and the distance within species was generally less than 2%. However, some species presented a COI variation far superior to 10% (e.g., *T. tubifex*: 20.7%), which demonstrated the existence of cryptic species. Sturmbauer et al. (1999) and Beauchamp et al. (2001) studied the molecular phylogeny of *T. tubifex* in Northern Europe and North America respectively, and found that the maximum genetic distance of mitochondrial 16S gene was greater than 10%. The 16S gene is more conserved than COI gene, and this value revealed a big difference within *T. tubifex*, which proved the existence of cryptic species. Genetic variation of COI gene from Europe and North America was researched in *L. variegatus* by Gustafsson et al. (2009). *Lumbriculus variegatus* was found to comprise two distinct clades (I and II), and the COI genetic distance was up to 17.7% between I and II. Gustafsson et al. considered that two clades were separate species.

Sequences can be divided quickly and effectively by ABGD method according to the principle of "DNA barcode gap" (Puillandre et al. 2011). The result of initial partition is more stable than recursive partition, which has been confirmed by related studies (Ratnasingham and Hebert 2013, Puillandre et al. 2011). In this study, all taxa were divided into 40 groups using the ABGD method, which was more than the results of morphology (36 species). One possible explanation was that the existence of cryptic species of *T. tubifex* and *L. variegatus*, which increased the number of groups. On the other hand, the COI gene, with higher evolutionary rate, is generally not enough to assess species boundaries alone (Achurra and Erséus 2013). Hence, the ITS2 gene was also used to increase reliability in our study. The ABGD delimitation model provided a consistent result with morphological observations basically.

The phylogenetic tree of ITS2 gene sorted all Naididae specimens into 3 branches: Tubificinae, Rhyacodrilinae and Naidinae. Because the phylogenetic relationship within the subfamily Rhyacodrilinae was still confused, the genus *Rhyacodrilus* was clustered in the subfamily Naidinae, so both subfamilies are not monophyletic. The genus *Nais* was non-monophyletic, and formed two clades. One clade comprised *N. badia*, *N. elinguis*, *Stylaria fossularis*, and *Slavina appendiculata* and the other consisting of the remaining *Nais* species and *Uncinais uncinata*. A recent finding revealed that a tropical freshwater origin of Naidinae, the phylogenetic relationships using combined markers by Bayesian inference supported that *Nais* was paraphyletic, and *Uncinais* was included in it (Erséus et al. 2017). *Nais badia*, endemic to Tibet, was sister to the clade consisting of *Stylaria fossularis* and *Slavina appendicula*. We guess it is because of pigments in the anterior segments of *N. badia* and *S. fossularis*. The genus *Tubifex* is not monophyletic. Many works have revealed

that there are cryptic species within *T. tubifex* (Sturmbauer et al. 1999). In the ITS2 tree, *Tubifex conicus* and *T. laxus* were clustered together *Isochaetides palmatus*, but not with *T. tubifex*. Therefore, the taxonomic status of *T. conicus* and *T. laxus* still need to be determined in the future. *Tubifex tubifex* and *T. blanchardi* are sisters. Chapman and Brinkhurst (1987) suggested that the hair chaetae of *T. tubifex* would be induced by the environment to disappear and become *T. blanchardi*, which were actually the same species. Crottini et al. (2008) suggested the variation of *T. blanchardi* and *T. tubifex* in the Lambro River based on the 16S gene and pointed out that *T. blanchardi* and *T. tubifex* were two independent lineages. Marotta et al. (2009) redescribed the morphological characteristics of *T. blanchardi* and found the differences of morphological with *T. tubifex*, thus finally confirming that *T. blanchardi* and *T. tubifex* are two separate species.

Conclusions

The study represents the first DNA barcoding study of the aquatic oligochaete in China, including endemic species and common species. Our findings detected that DNA barcoding based on COI gene is practicable and effective in identifying aquatic oligochaetes, however, combining ITS2 would be provide more information. The taxonomic status of some species needs to be confirmed through comprehensive taxonomic research, by expanding the sampling range and increasing the number of specimens. The barcodes of oligochaete species need to be added continuously to establish a native database so as to lay a solid foundation for the biological research of aquatic oligochaete.

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Author contributions

TTZ conceived the idea and wrote the first draft of the manuscript. YDC and HZW revised the manuscript and provided valuable suggestions. All authors read and approved the final version of the manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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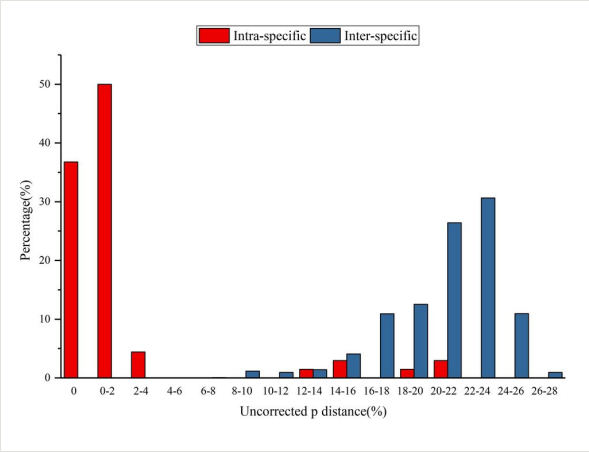

Figure 1.

Histogram of intra-specific and inter-specific genetic distances of COI gene from Naididae.

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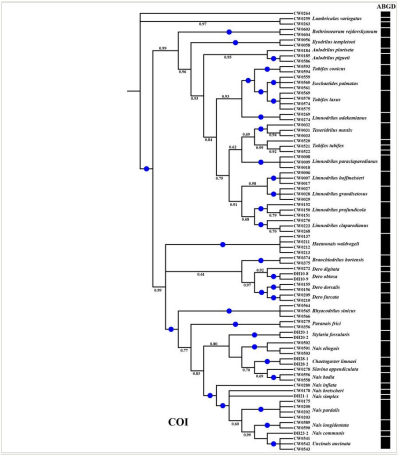

Figure 2.

Bayesian analysis of Naididae based on COI gene (Automatic partition results of ABGD). BI posterior probabilities > 0.60 are indicated and the circles represented 1.00.

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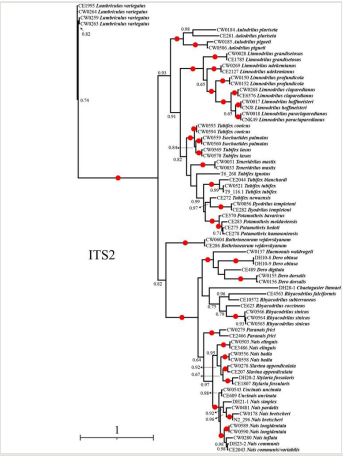

Figure 3.

Phylogenetic tree of Naididae based on ITS2 gene. BI posterior probabilities > 0.60 are indicated and the circles represented 1.00.

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Table 1.

Uncorrected pairwise distances of Naididae.

	Minimum (%)	Maximum (%)	Mean value (%)
Within species	0.0	20.7	1.9
Within genera	7.6	24.6	17.9
Within subfamilies	8.7	25.6	18.9

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Supplementary material

Suppl. material 1: Collection information of specimens of Naididae

Authors: Tingting Zhou, Hongzhu Wang and Yongde Cui
Data type: GenBank accession codes and collection information
Brief description: Collection information of specimens of Naididae. New sequences are shown in bold. Missing data is marked with "-".
Download file (45.73 kb)